Genetic identification of a respiratory arsenate reductase

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For more than a decade, it has been recognized that arsenate [$H_2AsO_4^{1-}$; As(V)] can be used by microorganisms as a terminal electron acceptor in anaerobic respiration. Given the toxicity of arsenic, the mechanistic basis of this process is intriguing, as is its evolutionary origin. Here we show that a two-gene cluster (*arrAB*; *a*rsenate *r*espiratory *r*eduction) in the bacterium *Shewanella* sp. strain ANA-3 specifically confers respiratory As(V) reductase activity. Mutants with in-frame deletions of either *arrA* or *arrB* are incapable of growing on As(V), yet both are able to grow on a wide variety of other electron acceptors as efficiently as the wild-type. Complementation by the wild-type sequence rescues the mutants' ability to respire As(V). *arrA* is predicted to encode a 95.2-kDa protein with sequence motifs similar to the molybdenum containing enzymes of the dimethyl sulfoxide reductase family. *arrB* is predicted to encode a 25.7-kDa iron–sulfur protein. *arrA* and *arrB* comprise an operon that contains a twin arginine translocation (Tat) motif in ArrA (but not in ArrB) as well as a putative anaerobic transcription factor binding site upstream of *arrA*, suggesting that the respiratory As(V) reductase is exported to the periplasm via the Tat pathway and under anaerobic transcriptional control. These genes appear to define a new class of reductases that are specific for respiratory As(V) reduction.

The consumption of arsenic (As)-tainted surface waters and ground waters has created a public health crisis in many countries (1, 2). Although much of the As contamination derives from natural weathering and dissolution of As-bearing minerals, recognition that microorganisms can alter the mobility of As in natural waters through redox transformations (3) drove the discovery of the first arsenate [As(V)]-respiring bacterium nearly a decade ago (4). Since then, many more microorganisms that can reduce As(V) to arsenite [H_3AsO_3, As(III)] have been discovered (5–8), but a mechanistic understanding of this metabolism has lagged. To date, only three studies have described the biochemistry of arsenate respiration (5, 9, 10), and detailed biochemical analyses have not been performed. In part, the limitations of these studies can be attributed to the fact that the As(V)-respiring organisms being studied were not genetically tractable. To quantify the geochemical impact of As(V)-respiring microorganisms in a given locale, we must be able to predict when these organisms will be active, and how rapidly they will transform As(V). Identification of the gene(s) that control this process, elucidation of their regulation, and determination of the kinetics of their protein products, are necessary steps toward understanding the specific contribution of As(V)-respiring bacteria to As-cycling in the environment.

In response to this need, a new As(V)-respiring species, *Shewanella* strain ANA-3 that is amenable to genetic analysis, was recently isolated (8). This organism contains two systems for reducing As(V). One is similar to the well conserved *ars* detoxification system from *Escherichia coli* plasmid R773 (11) and is advantageous, but not required, for respiratory As(V) reduction (8); a separate system appears to be required for As(V) respiration. In this report, we use genetic analysis to identify and describe the operon that encodes that latter system. Here we identify specific genes that are required for respiratory As(V) reduction, which opens the door for detailed biochemical study of their gene products. Moreover, the gene sequences we report represent a previously unrecognized class of respiratory reductases.

Materials and Methods

Strains and Plasmids. Bacterial strains of *E. coli* and *Shewanella* used in this study are listed in Table 1. Plasmids that were used or constructed are described in Table 1.

Growth Conditions. LB medium (12) was used for routine culturing of ANA-3 and *E. coli* strains. Anaerobic growth medium for strains of ANA-3 consisted of the following minimal medium: 0.225 g/liter K_2HPO_4/0.225 g/liter KH_2PO_4/0.46 g/liter NaCl/0.225 g/liter $(NH_4)_2SO_4$/0.117 g/liter $MgSO_4{\cdot}7H_2O$/4.2 g/liter $NaHCO_3$. The medium was supplemented with 20 mM sodium lactate, 10 mM Na_2HAsO_4, and 5 ml/liter each of trace element and vitamin solutions (8).

Identification of the Respiratory As(V) Reductase System. A partial *arrA* gene (1.2 kb) was identified on a previously isolated cosmid clone generated from genomic DNA of ANA-3 (8). Inverse PCR was used to isolate and sequence the downstream region of the partial *arrA* sequence. Genomic DNA (1 μg) was digested in a 50-μl volume with *Hin*dIII or *Bam*HI. After heat inactivation of the enzymes at 65°C for 20 min, intramolecular ligation reactions were performed: ≈400 ng of DNA was diluted to a concentration of ≈2 ng/μl and ligated with 2 μl of T4 DNA ligase (New England Biolabs) at 16°C overnight. After heat inactivation at 65°C for 15 min, reactions were adjusted to 500 μl with sterile water, applied to a Nanosep 30K centrifugal device (Pall Gelman Laboratory), and concentrated to ≈40 μl according to the manufacturer's instructions. PCR was performed with 2 μl of concentrated ligation reactions as follows: 200 nM of each inverse primer ARR-R1 (5′-CACCATTCGACTAAACCGTAGG-3′) and ARR-L1 (5′-GCGAAAGCCTATATGGATGAGA-3′), 200 μM dNTP mix, 2.5 units of *Pfu*Turbo DNA polymerase (Stratagene), 1× *Pfu*Turbo reaction buffer in a 50-μl reaction volume. The PCR products were purified by using a PCR cleanup kit (Qiagen, Valencia, CA) and sequenced directly by primer walking. Sequence data were assembled by using TIGR ASSEMBLER (www.tigr.org/software/assembler), and ORFs were detected by using ORFINDER (www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted translation of each ORF was compared with the sequences within GenBank by using BLAST (www.ncbi.nlm.nih.gov/blast).

Mutagenesis. To determine whether *arrA* and *arrB* were required for As(V) respiration, two mutant strains of ANA-3 were made (ARRA3 and ARRB1) that contained in-frame deletions of the respective genes. Vectors pΔ*arrA*3 and pΔ*arrB*1 were con-

Abbreviations: As, arsenic; As(V), arsenate; As(III), arsenite; TMAO, trimethylamine-*N*-oxide.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY271310).

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MICROBIOLOGY

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or markers; characteristics and uses	Source or ref.
E. coli strains		
DH10β	Host for *E. coli* cloning; F-*mcrA* Δ*(mrr-hsdRMS-mcrBC)* Φ*80dlacZ*Δ*M15* Δ*(codB-lacI)3 deoR recA1 endA1 araD139* Δ*(ara-leu)7697 galU galK* λ*-rpsL(*StrR*)*	Life Technology
UQ950	*E. coli* DH5α λ(pir) host for cloning; F-Δ*(argF-lac)169* Φ*80dlacZ58(*ΔM15) *glnV44*(AS) *rfbD1 gyrA96*(NalR) *recA1 endA1 spoT1 thi-1 hsdR17 deoR* λ*pir+*	D. Lies, Caltech
WM3064	Donor strain for conjugation: *thrB*1004 *pro thi rpsL hsdS lacZ*ΔM15 RP4–1360 Δ(*araBAD)567* Δ*dapA*1341::[*erm pir*(wt)]	W. Metcalf, Univ. of Illinois, Urbana
Shewanella strains		
ANA-3	Isolated from an As-treated wooden pier pilling in a brackish estuary (Eel Pond, Woods Hole, MA); Contains *arsDABC*; respires on As(V); resistant to > 5 mM arsenite.	8
ARRA3	*Shewanella* sp. str. ANA-3, Δ*arrA*3; does not respire As(V)	This study
ARRB1	*Shewanella* sp. str. ANA-3, Δ*arrB*1; does not respire As(V)	This study
Plasmids/vectors		
pSALT1	pLAFR5-based 45 kb As(III)^r cosmid from ANA-3 genomic DNA library; contains *arsDABC*, Tc^r, confers resistance to As(III) and As(V).	8
pSMV10	9.1-kb mobilizable suicide vector; *oriR6K*, *mobRP4*, *sacB*, Km^r Gm^r	D. Lies, Caltech
pΔ*arrA3*	2-kb fusion PCR fragment containing Δ*arrA* cloned into the *Spe*I site of pSMV10; used to make the ARRA3 Δ*arrA* strain.	This study
pΔ*arrBI*	2-kb fusion PCR fragment containing Δ*arrB* cloned into the *Spe*I site of pSMV10; used to make the ARRBI Δ*arrB* strain.	This study
pBBR1MCS-2	5.1-kb broad-host range plasmid: Km^r, *lacZ*	15
*parrA*4	*arrA4* PCR fragment, including the promoter region, cloned into the *Spe*I site of pBBR1MCS-2	This study
*parrB*1	*arrB1* PCR fragment cloned into the *Xho*I/*Spe*I sites of pBBR1MCS-2	This study

structed by a cross-over PCR technique (13). Fusion PCR products (with *Spe*I ends, underlined), generated by using *Pfu*-Turbo (Stratagene), were prepared in two sequential PCRs that involved amplification of 1-kb regions up- and downstream of *arrA* with primers: XARR-A-A (5′-GGACTAGTGTGAGTCCAGCAACGCTAT), XARR-A-B (5′-CCCATCCAGCATGCTTAAACAGACTTGATTCTCTTTCTTCATTTTC), XARR-A-C (5′-TGTTTAAGCATGCTGGATGGGCGTGTTGAGAAAGTGTGAGGT), and XARR-A-D (5′-GGACTAGTATGACTTGATCCCTGAAATTG). For *arrB* the primers were: XARR-B-A (5′-GGACTAGTATTCTATCGGTAATGGTGTCG), XARR-B-B (5′-CCCATCCAGCATGCTTAAACACATTCCTAATCTCATAGTGGG), XARR-B-C (5′-TGTTTAAGCATGCTGGATGGGAAAACCGCTTATTAATCATAGAGG), and XARR-B-D (5′-GGACTAGTGCATAGCAACAGCAACCTTT). The two 1-kb flanking PCR products for each gene deletion were purified by using a Qiagen PCR clean-up column and mixed together to perform a second PCR with primers XARR-A-A and XARR-A-D (for *arrA*), or XARR-B-A and XARR-B-D (for *arrB*). The resulting 2-kb fusion products were digested with *Spe*I and ligated into the *Spe*I site of pSMV10, and transformed into a DH5α *λpir* strain (UQ950). The resulting mutagenesis vectors were transformed into the plasmid donor strain, *E. coli* WM3064 (a *dap* auxotroph, derivative of strain B2155; ref. 14), grown on LB + 0.3 mM diaminopimelic acid (DAP), and transferred to ANA-3 by conjugation (2:1 donor/recipient ratio; 5 h mating on a LB/DAP plate at 30°C). ANA-3 transconjugants containing the integrated mutagenesis vector were selected on LB agar plates supplemented with 50 μg/ml kanamycin (8). Kanamycin-resistant colonies occurred at frequencies ranging from 10^{-8} to 10^{-9} per recipient. These colonies were grown in the absence of antibiotic and subcultured once into LB. After overnight growth at 30°C, dilutions were plated on LB/10% sucrose (to select for loss of the *sacB* gene on the integrated mutagenesis vector), dried in a laminar flow hood, and incubated at 30°C overnight. Plates were replica printed onto LB and LB/kanamycin agar. Kanamycin-sensitive colonies were screened by PCR for the deletion of *arrA* or *arrB*.

Plasmids p*arrA*4 and p*arrB*1 were constructed to complement the ARRA3 and ARRB1 *arr* deletion mutants. PCR was used to generate *arrA*4 [*Spe*I ArrA-F1 (5′-GGACTAGTGAATAGGAGGCGATAAATGGAG), *Spe*I ArrA-R1 (5′-GGACTAGTCCTAATCTCATAGTGGGTTACCTC)] and *arrB*1 [*Xho*I ArrB-F1 (5′-GGCTCGAGGAAAGTGTGAGGTAACCCA), *Spe*I ArrB-R1 (5′-GGACTAGTCTCTATGATTAATAAGCGGT)] gene fragments, which were then ligated to the broad-host range plasmid pBBR1MCS-2 (15) to generate the complementation vectors. The appropriate vector was mated into the ANA-3 *arr* deletion strains by using *E. coli* WM3064 as the donor; transconjugants were selected by plating on LB agar supplemented with 50 μg/ml kanamycin without diaminopimelic acid. Kanamycin-resistant colonies were streak-purified several times on LB agar plates containing kanamycin.

A microtiter dish assay was used to test whether the *arr* deletion strains could grow with other terminal electron acceptors including fumarate (20 mM), nitrate (20 mM), thiosulfate (20 mM), trimethylamine-*N*-oxide (TMAO) (10 mM), poorly crystalline manganese oxides and iron hydroxides (20 mM) (8), and 2,6-anthraquinonedisulfonate (5 mM). Approximately 10^8 cells of overnight LB-grown cultures were washed two times in 1× PBS (12). A total of 200 μl of anaerobic media for each electron donor were inoculated in quadruplicate wells of a microtiter plate at 10^6 cells per ml. Microtiter plates were incubated at 30°C in an anaerobic chamber (15% CO_2/80% N_2/5% H_2). Growth was monitored over several days by observing the increase in the optical density at 600 nm. For metal oxides, utilization of the electron acceptors was inferred by observing the darkening (from red to black) of the iron oxide or the clearing (from black to clear) of the manganese oxide in the plate. Controls without electron donors and/or cells were also included.

Time Course of Respiratory Growth on Arsenate. Strains of ANA-3 were grown in LB or LB/kanamycin overnight at 30°C. Cells were washed two times in 1× PBS and resuspended to a final density of $\approx 10^8$ cells per ml. Washed cells were inoculated into 10 ml of As(V)/lactate minimal salts medium at a cell density of

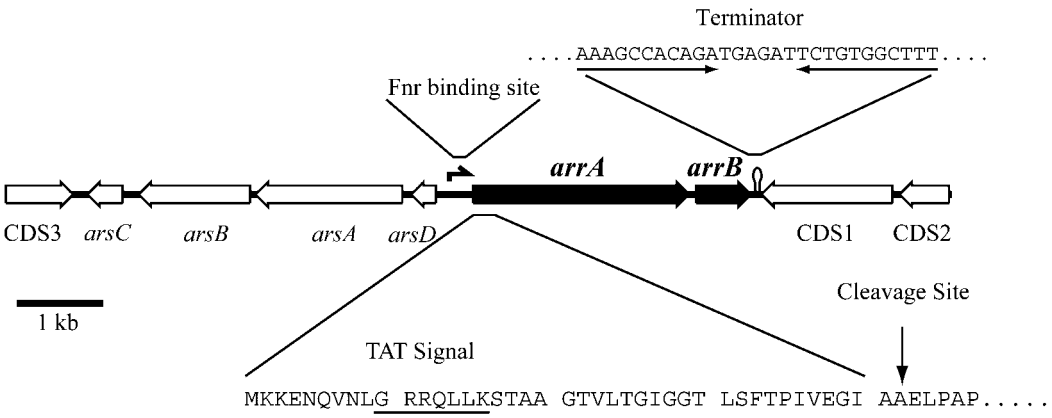

Fig. 1. Molecular organization of the *arrAB* gene cluster. The *arrA* gene is located upstream in the opposite orientation to an *ars* operon. CDS1 and CDS2 are similar to glutathione synthetases and a *S. oneidensis* MR-1 conserved hypothetical protein, respectively. The arrow between *arsD* and *arrA* indicates the location of potential Fnr-like binding sites. The loop after *arrB* is a putative transcriptional terminator region. Analysis of the putative Tat signal sequence within the first 42 amino acid residues of ArrA reveals a potential cleavage site (arrow).

$\approx 10^6$ per ml and incubated at 30°C. Cultures were sampled periodically and analyzed for cell growth, As(V) reduction, and lactate oxidation.

Phylogenetic Analysis. Sequences were aligned with CLUSTALW 1.82 (16). Phylogenetic analyses were performed by using the program PAUP* 4.0b10 (17). The distance criterion was used to construct unrooted neighbor-joining trees. After ignoring gaps in the multisequence alignments for ArrA and ArrB, the final number of amino acids included in the phylogenies were 510 and 144, respectively.

Analytical Techniques. As(V), As(III), lactate, and acetate were quantified by high-performance liquid chromatography (HPLC) as described (8). Changes in culture optical density were monitored at 600 nm.

Results

Identification of the *arrAB* Gene Cluster. Previously, we identified a region of DNA that encoded an arsenic resistance operon, *arsDABC*, from a genomic library of ANA-3 (8). Sequencing upstream of this cosmid clone (pSALT1) revealed a partial gene fragment (*arrA*) whose predicted amino acid sequence indicated that it had a molybdopterin cofactor binding site. This sequence homology suggested that the *arrA* gene might encode a component of an anaerobic reductase, and that this reductase might be the respiratory As(V)-reductase. We therefore sequenced the DNA downstream of the partial *arrA* fragment. Fig. 1 shows a map of the predicted ORFs. The complete *arrA* ORF is followed by another ORF, *arrB,* immediately downstream of *arrA*. Two other predicted coding sequences with opposite orientations to *arrAB* also occur downstream (CDS1 and CDS2). BLAST analyses of CDS1 and CDS2 showed that they were similar to glutathione synthases and a conserved hypothetical protein found in *Shewanella oneidensis* strain MR-1, respectively. Two putative Fnr-like binding motifs TTGAT-(N_{14})-ATAAA and TTGAT-(N_{12})-AGCAA (18) exist between *arsD* and *arrA*. A potential transcriptional terminator 32 bp downstream of *arrB* was also identified (AAAGCCACAGATGAGATTCTGTGGCTTT; inverted repeats underlined). Ribosome binding sites occur 13 bp upstream of *arrA* (AGGAG) and 13 bp upstream of *arrB* (GAGG). These sequence features suggest that *arrA* and *arrB* comprise an operon.

Physiology of the Mutants. To test the prediction that the *arrAB* gene cluster encodes the respiratory As(V) reductase, two strains of ANA-3 with nonpolar deletions in either *arrA* or *arrB* were constructed. Fig. 2 shows the respiratory growth response of strain ARRA3 (Δ*arrA*) and strain ARRB1 (Δ*arrB*) in comparison to the wild type when grown anaerobically with As(V) as the terminal electron acceptor. Whereas the wild type completely reduced As(V) to As(III) and reached stationary phase within 24 h, the deletion strains were unable to grow (Fig. 2*A*) and unable to reduce As(V) to As(III) (Fig. 2*B*) under these conditions. When ARRA3 and ARRB1 were complemented by either p*arrA4* or p*arrB1*, respectively, they regained their ability to grow and reduce As(V) under anaerobic conditions (Fig. 2*C*). The cell density for wild-type ANA-3 generally remains at $\approx 10^8$ cells per ml for several days (8); however, microscopic observation reveals that the cells shrink in size after reaching stationary phase. Consequently, the decline in optical density observed over time (see Fig. 2) most likely reflects this phenomenon.

To determine whether the mutants were specifically defective in respiratory electron transfer to As(V), we checked their ability to reduce other electron acceptors, including oxygen, fumarate, nitrate, poorly crystalline manganese oxide, poorly crystalline iron oxide, TMAO, thiosulfate, and the humic substance analog, anthraquinone disulfonate (AQDS). In all cases except for As(V), strain ARRA3 and strain ARRB1 were positive for growth and/or reduction similar to wild-type ANA-3 (8).

Sequence Analysis of *arrA* and *arrB*. The predicted molecular mass of the *arrA* gene product is 95.2 kDa and its predicted pI is 9. Closer inspection of the predicted amino acid sequence of ArrA suggests that it shares several common features of bacterial molybdopterin oxidoreductases. It contains a cysteine-rich motif (C-X_2-C-X_3-C-X_{27}-C) beginning at amino acid residue 61 and extending to position 96 that is predicted to coordinate an iron–sulfur cluster, a molybdopterin dinucleotide-binding domain (residues 715–837), and a twin arginine signal sequence (S/T-R-R-X-F-L-K) (residues 10–16) (19). Twin arginine signal sequences are commonly found at the N-terminal region of periplasmic oxidoreductases (e.g., the *Shewanella massilia* TMAO reductase (20) and the *E. coli* periplasmic nitrate reductase, NapA (21, 22)). When the signal peptide prediction program SIGNALP (www.cbs.dtu.dk/services/SignalP/) was used, a Tat recognition region, followed by a hydrophobic and a weakly charged region, were found in the first 42 aa of ArrA. These residues might comprise the part of the protein that crosses the cytoplasmic membrane, consistent with the identification of a potential cleavage site at Ala-42 detected by SIGNALP (indicated by an arrow in Fig. 1). In contrast, the *arrB* gene is directly

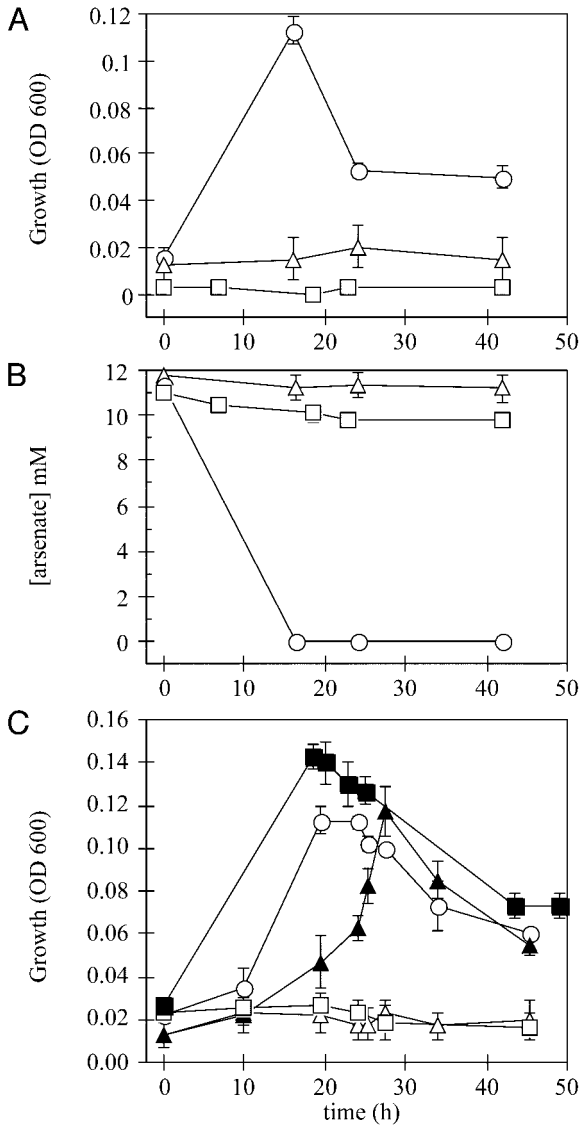

Fig. 2. As(V) respiration by wild-type ANA-3 (circles) and mutant strains ARRA3 (Δ*arrA*) (squares) and ARRB1 (Δ*arrB*) (triangles). (*A*) The time course for growth inferred by optical density at 600 nm. (*B*) The As(V) concentration in the medium at the various time points. (*C*) Anaerobic growth on As(V) by strain ARRA3 (Δ*arrA*) and strain ARRB1 (Δ*arrB*) is restored by providing a wild-type copy of the *arrA* or *arrB* gene on a complementation vector (filled symbols). Open symbols in *C* represent strains containing the complementation vector alone. Points and error bars in each panel represent the averages and standard deviations of triplicate samples.

downstream of *arrA* and is predicted to encode a protein that has a molecular mass of 25.7 kDa and a pI of 9. Sequence analysis of ArrB indicates that it could contain four iron–sulfur clusters at the following cysteine residues: (*i*) cys12, 15, 18, 69; (*ii*) cys 22, 60, 65, 57; (*iii*) cys 89, 92, 95, 183; and (*iv*) cys 99, 164, 167, 179. The arrangement of the four putative iron–sulfur clusters are predicted to be similar to the model proposed for the *E. coli* DMSO reductase iron–sulfur subunit, DmsB (23). ArrB does not contain a Tat signal sequence.

Phylogenetic Analysis of ArrA and ArrB. To gain insight into the evolutionary history of *arrA* and *arrB*, phylogenetic trees of their predicted protein products were constructed. Fig. 3*A* shows the phylogenetic relationship of ArrA to representative members of the dimethyl sulfoxide reductase family of molybdenum containing enzymes (19), including periplasmic and membrane-associated nitrate reductases (NapA and NarG), TMAO and DMSO reductases (TorA/DorA and DmsA), biotinsulfoxide reductases (BisC), formate dehydrogenases (FdhF/A/G), selenate reductase (SerA), arsenite oxidase (AsoA), and polysulfide reductase (PsrA/PhsA). ArrA clusters most closely with a hypothetical protein from the As(V)-respiring bacterium *Desulfitobacterium hafniense* (Desu0744; 51% amino acid identity, 68% amino acid similarity) and a putative arsenate reductase from *Bacillus selenitireducens* (ref. 24; 46% amino acid identity, 63% amino acid similarity).

Fig. 3*B* shows the phylogenetic relationship of ArrB to respiratory proteins containing iron–sulfur subunits including SdhB (succinate dehydrogenase), FrdB (fumarate reductase), NrfC (nitrite reductase), PsrB/PhsB, DmsB, and NarH. ArrB forms a cluster with NrfC and PrsB/PhsB. ArrB is most similar to a hypothetical iron–sulfur protein from *D. hafniense* (Desu0743; 53% amino acid identity, 61% amino acid similarity). As in strain ANA-3, the ORF encoding the ArrB homolog in *D. hafniense* is located immediately downstream of the ORF that encodes the ArrA homolog.

Discussion

When genetic analysis was used, a new gene cluster that encodes a respiratory As(V) reductase in the *Shewanella* species strain ANA-3 was identified. The two genes in this cluster, *arrA* and *arrB*, encode proteins that are predicted to be similar to those involved in the respiratory reduction of DMSO, polysulfide, TMAO, selenate, and nitrate, as well as the oxidation of formate and arsenite. Specifically, ArrA is predicted to bind a molybdenum cofactor, and ArrB is predicted to contain iron–sulfur cultures. A Tat motif at the N terminus of ArrA suggests that the As(V) reductase complex is exported to the periplasm, consistent with the fact that molybdopterin-containing proteins often are exported by the Tat pathway in their folded state (25). A Tat motif is also present in the sequence of the ArrA homolog from *D. hafniense.*

Detailed biochemical studies will be needed to describe the exact mechanism of respiratory electron transfer to As(V), but it is clear from our sequence data that the mechanism will be unlike that used to reduce As(V) by the detoxifying ArsC enzymes (11). Although the detoxifying and respiratory As(V) reductases share a common reaction substrate and product, they are completely different in structure and function. In the case of the ArsC enzymes, specific thiol groups are known to transfer electrons from intracellular reductants like glutathione or thioredoxin to As(V) in a redox cascade and no energy is gained from this process (11). In the case of the ArrA/ArrB respiratory reductase from ANA-3, a periplasmic protein complex containing a molybdopterin subunit and iron–sulfur clusters would appear to accept electrons delivered by c-type cytochromes in the cytoplasmic membrane. This is reminiscent of the type of electron transfer system thought to be involved in the oxidation of As(III) (11, 26), but with reverse electron flow. The apparent electron transfer scheme of the respiratory As(V) reductase is basically that of a classic bioenergetic chain for anaerobic respiration.

The discovery that the genes encoding the second As(V) reductase in ANA-3 are similar to those that encode enzymes involved in other types of anaerobic respiration is not surprising. What is interesting, however, is that these genes appear to define a new class of reductases that are specific for respiration on As(V); moreover, these genes appear to have a broad phylogenetic distribution. A previous report described the purification of a respiratory As(V) reductase from *Chrysiogenes arsenatis* that couples the oxidation of methyl viologen to the reduction of As(V) to As(III) (9). This enzyme has two subunits: an 87-kDa molybdenum-containing subunit (ArrA), and a 29-kDa iron–

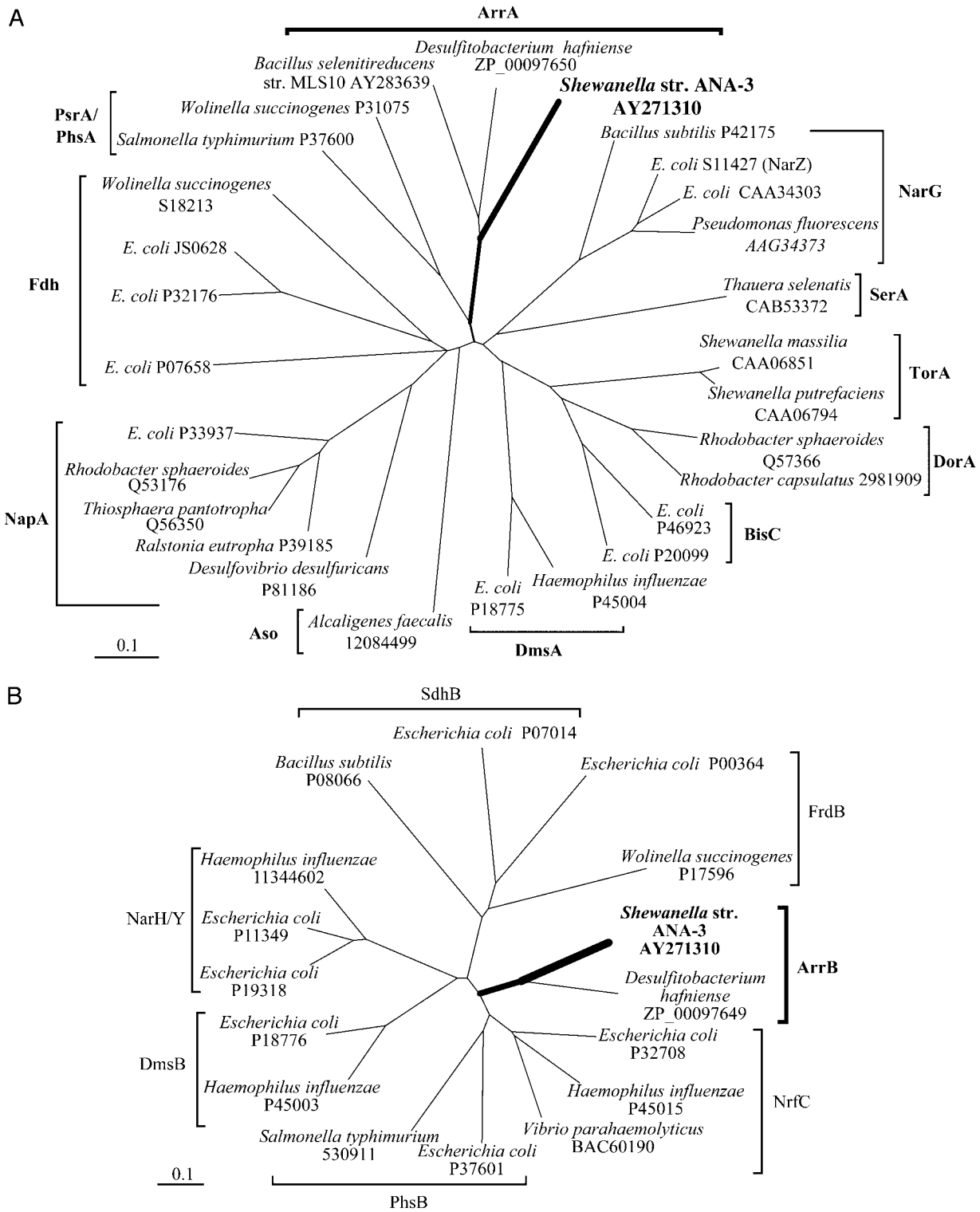

Fig. 3. Unrooted neighbor-joining trees for representative sequences from the DMSO reductase family of molybdoenzymes (19) (*A*) and iron–sulfur proteins (*B*). Aso, arsenite oxidase; Fdh, formate dehydrogenase; Nap, periplasmic nitrate reductase; Dor/Dms, DMSO reductase; Bis, biotinsulfoxide reductase; Tor, trimethylamineoxide reductase; Ser, selenate reductase; Nar, membrane-associated nitrate reductase; Arr, arsenate respiratory reductase; Psr/Phs, polysulfide reductase; Sdh, succinate dehydrogenase; Frd, fumarate reductase; Nrf, nitrite reductase. GenBank accession numbers are included next to the corresponding species. Scale bar represents the number of amino acid changes per site.

sulfur-containing subunit (ArrB). When we compare the N-terminal sequences of the *C. arsenatis* ArrA and ArrB proteins to the predicted N-terminal sequences of ArrA and ArrB from strain ANA-3, we find that they are more similar to each other than they are to any other known proteins. Furthermore, two hypothetical proteins in the genome sequence of the As(V) respiring bacterium, *D. hafniense* (7), are strikingly similar to ArrA and ArrB from ANA-3, suggesting that they too might

encode components of a respiratory As(V) reductase. Biochemical and molecular data emerging from studies of the haloalkaliphilic As(V) respiring bacterium *B. selenitireducens* strain MLS10, also suggest that ArrA and ArrB homologs are present in this strain (24). These organisms are only very distantly related to each other, representing three distinct phyla within the *Bacteria* (27): *Shewanella* ANA-3 (8) is a member of the γ-subgroup of the *Proteobacteria*, *C. arsenatis* (28) is considered the type species for the *Chrysiogenetes*, whereas *D. hafniense* (29) and *B. selenitireducens* (30) both represent the phylum *Firmicutes*. It is possible, therefore, that the *arrA* and *arrB* genes might have been distributed through horizontal gene transfer; alternatively, they may be evolutionarily deep. Before drawing conclusions about the evolution of these genes and/or how they may be used to design molecular probes to detect As(V) respiratory activity in the environment, more genetic studies describing functional arsenate-reductases from other arsenate-respiring organisms will be necessary.

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